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13. ABSTRACT *(Maximum 200 words)*

RPTPα is a protein tyrosine phosphatase implicated in the activation of Src family kinases, and in the regulation of integrin signaling, cell adhesion, and growth factor responsiveness. To explore its potential contribution to human neoplasia, we surveyed RPTPα protein levels in 51 primary breast cancer samples. We found RPTPα expression to vary widely among indidvidual tumors, with significant overexpression occurring in approx. 27% of cases. RPTPα overexpression reflected reduced tumor aggressiveness, being strongly negatively correlated with tumor grade. In cell culture, expression of RPTPα in MCF-7 breast carcinoma cells led to growth inhibition, associated with increased accumulation in the G1 phase of the cell cycle. RPTPα expression also resulted in delayed tumor growth and metastasis in nude mice tumorigenicity assays. We propose that RPTPα overexpression in breast cancer constitutes a secundary response by which the cell attempts to maintain homeostasis perturbed during neoplastic transformation. To our knowledge, this is the first example of a study correlating expression level of a *bona fide* protein tyrosine phosphatase with neoplastic disease in humans.

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Jon Soyn 05/27/99

PI - Signature Date

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Introduction

It has become clear that signaling by tyrosine kinases is tightly controlled by, and intimately linked to, the function of protein phosphatases. This proposal was aimed at analyzing the role of RPTPα-like PTPases in breast tumors. Studies in fibroblasts and PC12 cells respectively had demonstrated that RPTPα acts as an activator of Src family kinases (SFKs), and can potentiate signaling through the EGF-receptor. In preliminary data RPTPα or its closest relative, RPTPε, were found overexpressed in a subset of mammary tumors. We therefore hypothesized that RPTPα expression might be selected for and potentiate or mimic the function of the the HER2/neu oncogene in tumorgenesis. In this proposal, we wanted to test this hypothesis by 1) analyzing the correlation between RPTPα expression and other tumor markers, 2) performing *in vitro* studies using mammary cell lines transfected with RPTPα, and 3) studying tumor induction in MMTV-RPTPα transgenic mice.

Body

Progress is discussed here according to the original subdivisions in the statement of work. Most belonging under aims 1 and 2 (except months 10-12) are described in full detail in a manuscipt by Ardini *et al.* (see appendix) which is about to be submitted.

Months 1-3

-finalization of aim 1 PCR and immunohistochemical analysis to estimate the incidence of increased RPTPα (or RPTPα -related proteins} overexpression in human breast tumors

PCR analysis has been replaced by Western analysis (of protein lysates), and Northern and Southern analysis (on RNA and DNA respectively) since these give the same, but more quantitative conclusions than PCR or immunohistochemistry. The results demonstrated a negative correlation between RPTPα overexpression and tumor grade (appended manuscript by Ardini *et al.*, table 1).

-aim 2: construction of erbB2 expression vectors, construction of vectors for inducible RPTPα expression.

These vectors have been constructed.

-aim 3: finalization and testing (by transient expression in cell lines) of the MMTV-RPTPα expression vector.

The vector has been tested and shown to work.

Months 4-9

-aim 1: preparation of manuscript describing the results of the analysis of RPTPα (or related PTPases) in human breast tumors

See the manuscript by Ardini *et al.*, included in the appendix.

-aim 2: generation of cell lines, and initial characterization

We have chosen to concentrate on RPTPα-overexpressing MCF7 cells. We have now generated a number of these lines, and have actually moved beyond generation of these cells to an initial characterization of their growth parameters. These experiments show that RPTPα expression is associated with increased c-Src kinase activity, and a reduced growth rates due to an expansion of G1.

-aim 3: start of zygote injections, and growth of mice

Performed (results see below)

Months 10-12

-aim 2: characterization of ligand binding properties of transfected cells.

This part of the proposal has been postponed, for 2 reasons: 1) We have chosen to initially perform a more detailed analysis of the effect of RPTPα expression by itself on the growth parameters of the cells (results described in the manuscipt by Ardini *et al.*; see appendix). 2) The tumor data suggest no correlation between RPTPα expression and HER2/neu status, making this aspect of the proposal less relevant.

-*aim 3:* testing of micro-injected mouse lines for transmission, and expansion of the colony

We tentatively have identified (by PCR) 3 positive transgenics, 2 of which are already known to transmit the transgenic allele.

Key research accomplishments

-RPTPα expression in tumor samples is significantly ($p<0.05$) associated with low grade tumors, and ER-positivity

-increased expression of RPTPα in MCF-7 cells results in reduced growth rates, and an enhanced accumulation in the G1 phase of the cell cycle.

-forced expression of RPTPα in mouse mammary N202.1A cells results in reduced tumorigenicity in nude mice assays.

Reportable outcomes

"Expression of receptor protein tyrosine phosphatase alpha (RPTPα) in primary human breast cancer correlates with low tumor grade, and inhibits tumor cell growth *in vitro* and *in vivo* "
by E. Ardini, L. Yang, S. Menard, and J. Sap (1999, manuscript in preparation).
(SEE APPENDIX)

Conclusions

1. expression of RPTPα in primary tumors correlates significantly with grade.
2. expression of RPTPα in cultured mammary tumor cell lines results in G1 arrest in vitro, and reduced tumor growth and metastasis in nude mice assays.

Ensuing recommended changes for further work are:

1. perform a pilot analysis of the effect of RPTPα expression insulin and IGF-1 responsiveness.
2. work out conditions for immunochemistry using paraffin-embedded sections so that ultimately a larger number of cases can be screened with less effort.

Evaluation of knowledge as a scientific or medical product:

These pilot studies establish RPTPα expression in breast tumors as a potential marker for tumor progression or/and prognosis, which now hopefully will generate the attention to perform larger-scale studies on clinical cases.

References

see reference list in appended manuscript

Appendix

MANUSCRIPT IN PREPARATION:

Expression of receptor protein tyrosine phosphatase alpha (RPTPα) in primary human breast cancer correlates with low tumor grade, and inhibits tumor cell growth *in vitro* and *in vivo*.

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ABSTRACT

As a reversible process, tyrosine phosphorylation is controlled by the opposite activities of protein tyrosine kinases (PTKs) and phosphatases (PTPs). Whereas the contribution of PTKs to human breast tumorigenesis, and their relevance as prognostic markers, is the subject of intense scrutiny, the potential role of PTPases is almost completely unknown. RPTPα is a protein tyrosine phosphatase implicated in the activation of Src family kinases, and in the regulation of integrin signaling, cell adhesion, and growth factor responsiveness. To explore its potential contribution to human neoplasia, we surveyed RPTPα protein levels in 51 primary human breast cancer samples obtained at surgery. We found RPTPα expression to vary widely among indidvidual tumors, with significant overexpression occurring in approx. 27% of cases. RPTPα overexpression reflected reduced tumor aggressiveness, being negatively correlated with tumor grade. In cell culture, expression of RPTPα in MCF-7 breast carcinoma cells led to growth inhibition, associated with increased accumulation in the G1 phase of the cell cycle. RPTPα expression also resulted in delayed tumor growth and metastasis in nude mice tumorigenicity assays. We propose that RPTPα overexpression in breast cancer constitutes a secundary response by which the cell attempts to maintain homeostasis perturbed during neoplastic transformation. To our knowledge, this is the first example of a study correlating expression level of a *bona fide* protein tyrosine phosphatase with neoplastic disease in humans.

INTRODUCTION

Protein tyrosine phosphorylation is controlled by the opposite activity of protein tyrosine kinases (PTKs) and phosphatases (PTPases). The crucial involvement of tyrosine phosphorylation in control of cell growth, motility, and invasiveness is reflected in the contribution of many tyrosine kinases to neoplastic transformation [1]. In the case of breast cancer, overexpression of HER2/neu in particular has assumed the status of an important prognostic indicator, and may constitute a useful therapeutical target [2]. Other receptor tyrosine kinases (RTK) reported to be overexpressed in mammary tumors are *met* [3,4], *DDR* [5], and *ron* [6]. The IGF1-receptor is also amplified in some cases; the importance of insulin-like growth factors is thought to reside in their anti-apoptotic properties na their ability to affect estrogen-sensitivity [7]. In addition, increased enzymatic activity of (non-receptor) Src-family kinases has been recognized for a long time to be associated with neoplastic progression [8-10].

The biological activity of tyrosine kinases such as the ones discussed above is highly context-dependent, and the identification of cellular modifiers of tyrosine-kinase initiated responses has therefore constituted a rewarding field of study. For instance, the downstream signalling pathways activated byHER2/neu, are co-determined by the identity of its heterodimerization partners within the erbB family [11]. Two classes of trans-membrane glycoproteins were recently shown to act as intra-membrane modulators of erbB2 or EGF-receptor signalling [12,13]. The activity of Src-family kinases is itself controlled by the activation status of receptor tyrosine kinases, the non-receptor kinase *Csk*, as well as by protein tyrosine phosphatases (PTPases) [14].

Based on reversibility of tyrosine phosphorylation, protein-tyrosine phosphatases (PTPases) must be considered a potentially highly relevant family of signaling proteins altered expression or activity of which might contribute to neoplasia. Yet, their role in neoplastic transformation is almost completely unknown. The concept of clinically relevant

anti-oncogenically acting PTPases has received some support in the case of the PTEN/MMAC protein. However, the lipid phosphatase activity of the latter may actually be the crucial mediator of its biological function [15]. Clearly, however, PTPases can also act as positive regulators of signaling. They may cooperate with kinases by increasing substrate phosphorylation/dephosphorylation cycling rates [16]. SHP-2 is required for growth in response to some mitogens [17]. Studies on the PTPase CD45/LCA demonstrate its requirement for tyrosine phosphorylation and other signaling events upon T-cell receptor activation, due to its ability to act as an activator of Src-family kinases. However, inhibitory roles for SHP-2 and CD45 have also been described, and the function of PTPases in growth control may therefore also be highly lineage and signal-specific [18,19].

PTPases constitute a large family of structurally diverse enzymes [20]. Only a very limited number of studies so far have have addressed the possibility of deregulation of members of this family in breast cancer, or their contribution in breast cancer cell growth control. *In vitro* data suggest certain PTPases can inhibit growth of breast cancer cell lines [21,22]. PTPase activity raises when breast cancer cells are growth inhibited by antiestrogens [23]. In primary human tumors, membrane PTPase activity correlated with the presence of tumor positive axillary lymph nodes, whereas cytosolic PTPase activity correlated with the mitotic index [24]. Increased levels of RPTPε were observed in mouse tumors induced by transgenic HER2/neu or v-Ha-Ras, but not by c-myc or int-2 [25].

In this study, we focused our attention on the transmembrane (receptor) PTPase RPTPα. RPTPα is a widely expressed receptor-like protein tyrosine phosphatase displaying a short extracellular domain that is heavy glycosylated in the mature form of the protein, and a cytoplasmic region that contains two PTP domains, a membrane-proximal domain that is the most catalytically active, and a distal domain that might have a regulatory function. The ectodomain of RPTPα does not display features of typical cell adhesion molecules, such as

are found in many other receptor PTPases. RPTPα is expressed at low levels in most tissues or cell lines examined, with highest levels seen in brain or kidney [26].

Three features of RPTPα suggest it may play an important role in the regulation of cellular signalling pathways relevant to oncogenic transformation. First, it is found associated, via a tyrosine phosphorylation site in its C-terminus, with the adaptor protein Grb2 [27,28] . The existence of a RPTPα-Grb2 complex may implicate this PTPase in the control of Ras signaling. Second, overexpression of RPTPα is observed in advanced stage human colon carcinoma [29], while a closely related PTPase, RPTPε, was reported to be overexpressed in a subset of mouse mammary tumors [25]. Third, RPTPα appears to be an important regulator of the activity of Src family kinases. Its overexpression results in dephosphorylation of the carboxy-terminal negative regulatory Y527 site of c-src [30,31]. Intriguingly, activation of the kinase activity of c-Src is often observed in breast and colon tumor specimens [8,32], and can potentiate signalling by receptor tyrosine kinases of the ErbB family [33]. Conversely, genetic inactivation of RPTPα leads to reduced Src and Fyn function, and defects in integrin signalling [34].

The signaling properties of RPTPα to enhance the activity of c-src kinase and the possible involvement in Ras signalling, led us to hypothesize that in breast cancer RPTPα could act as a positive regulator of signalling and could play a role in neoplastic transformation or cancer progression.. To investigate the role of RPTPα in breast cancer we analyzed the expression of the molecule in 51 cases of primary breast tumors, and the effect of RPTPα gene transfection on *in vivo* and *in vitro* growth of breast carcinoma cell lines. The data obtained unexpectedly indicate that overexpression of RPTPα is associated with low proliferation and low aggressiveness.

MATERIALS AND METHODS

Human tumor samples

51 cases of fresh surgical tissues of pathologically confirmed primary breast carcinoma, or neighbouring non-diseased tissue from the same patient, were used in this study. All patients were enrolled at the National Cancer Institute of Milan for primary breast carcinoma, and underwent breast surgery with complete axillary dissection. Mean tumor size at pathological examination was 33.7 mm (range 15-80); according to TNM classification [35]. 10 patients were classified for tumor size as pT1 (<2 cm)(20%), 34 patients as pT2 (2-5 cm)(68%), and 6 patients as pT3 (>5 cm)(12%). 12 patients (23.5%) had no axillary lymph node involvement at histologic examination; among the other patients with pathologic nodal metastasis, 17 patients (43.6%) had a limited axillary metastatic involvement (1-4 metastatic nodes), whereas 22 patients (56.4%) had a high number of metastatic nodes. All patients had infiltrating carcinoma: 39 patients (76.5%) had infiltrating ductal carcinoma, 7 patients (13.7%) infiltrating lobular carcinoma, whereas the remaining 5 patients (9.8 %) had a less frequent histotype.

All human tissues were collected within 5 min of surgical resection, snap frozen in liquid nitrogen and stored at –80 °C until use. Primary tumor diameter and axillary nodal status were obtained from histopathological reports. Hematoxylin/eosin-stained histologic slides of each patient were reviewed for diagnostic reassessment of the histotype, grading, necrosis, proliferation index. Histologic grading was performed according to Elston, considering tubule formation, nuclear morphology and number of mitoses [36,37]. The latter finding was scored as "+" in case of more than 1 mitosis per high power field and "-" in case of 1 or less mitosis per high power field after analysis of at least 10 microscopic fields at 400x magnification. Proliferation index was evaluated by ^{3}H thymidine incorporation as previously reported, with 2.3% of labeled cells being the cut-off value [38].

Immunoblotting analysis

The specimens were homogenized and lysed for 1 h on ice in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were clarified for 15 min at 15000 g. Protein concentration was evaluated using the Biorad protein reagent assay. For each sample, 100 μg of total protein extract were loaded on a Laemmli 10% SDS-PAGE gel. For immunoprecipitation, Protein A-Sepharose was incubated with antibody for 1 h at 4 °C, washed three times with RIPA buffer and then incubated with 1 mg of total protein lysate for 2 h at 4 °C. Immune complexes were washed three times with RIPA buffer and eluted by heating for 5 min at 95 °C in Laemmli buffer. Proteins were separated by 10% SDS-PAGE and electophoretically transferred to a nitrocellulose membrane (Hybond C, Amersham). After overnight blocking in 5% low fat milk, the membrane was incubated for 2 hours with primary antibody followed by incubation with HRP-conjugated Protein A. Antibody binding was visualized using an ECL detection system (Amersham). Anti-RPTPα antibodies used were: 443, raised against the entire RPTPα intracellular domain; 35, against the RPTPα C-terminus (residues 785-802); and 210, against residues 518-531 (numbering according to [39]).

Southern Blot analysis

Genomic DNA from tumor and normal tissues was extracted using the SDS-Proteinase K method, digested overnight with EcoRI, then run on 0.8% agarose gel and transferred to nitrocellulose (Amersham). The probe fragment, spanning nucleotides 778 to 1107 [39], corresponding to the extracellular domain, was prepared by digestion with EcoNI of the human cDNA, and labelled with α-^{32}P-dCTP using a random primer labeling kit (Boehringer).

Cells and culture conditions

Human breast carcinoma MCF-7 cells and human kidney 293T cells were obtained from ATCC (Rockville, MD). The mouse mammary carcinoma cell line N202.1A (derived from HER2/neu protooncogene transgenic mice) was kindly provided by Dr. P.L. Lollini [40]. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 in air, and routinely cultured in DMEM supplemented with 10% FCS. MCF-7 cells transfected with pcDNA3/neo and with pcDNA3/RPTPα(*wt*) were cultured in the presence of 500 μg/ml G-418 (Life Technologies, Inc. Gaithesburg, MD). N202 infected with pLXSHD-derived virus were cultured in the presence of 4 mM histidinol (Sigma).

Plasmids

Full length mouse RPTPα cDNA was excised from Bluescript by digestion with XhoI and ThaI, and subcloned between the Xho I and Eco RV sites of the CMV early promoter-based expression vector pcDNA3/neo (Invitrogen). The retroviral construct in pLXSHD was described previously [27].

Infection of N202.1A cells

Retroviral constructs in pLXSHD were cotransfected into 293T cells together with a packaging-defective ecotropic helper construct [27], using calcium phosphate-mediated transfection. Conditioned medium was collected after 24 h, and contained virus titers (as assayed by the number of histidinol-resistant colonies in 3T3 cells) of approximately 10^5 colony-forming units/ml. Retrovirus-infected N202.1A cells were generated by infection with an appropriately low virus titer (pLXSHD-RPTPα(*wt*), or empty vector) for 3 h in the presence of polybrene (8 μg/ml) followed by histidinol selection (4 mM) starting 2 days after infection. To avoid artefacts of clonality, pooled resistant clones were used for tumorigenicity assays in nude mice injection.

Tumorigenicity and Experimental Metastasis Assay

In vivo experiments were conducted in six-week-old athymic mice purchased from Charles River (Calco, Italy). Care and use of the animals was in accordance with institutional guidelines. Mice (5 animals/cell type) were injected subcutaneously in the right flank with 300,000 cells each, and tumors calibrated every week. Tumor volume was calculated using the formula $0.5 \times d1^2 \times d2$ (where d1 and d2 are the larger and the smaller diameter, respectively). For experimental metastasis evaluation, mice were injected in the tail vein with 1×10^6 cells each (5 animals/cell type), and examined every 3 days. The presence of multiple lung metastases was evaluated at necropsy.

Statistical analysis

Data were evaluated using the Student's t-test. Differences were considered significant at $p<0.05$.

Transfection

MCF-7 cells were plated in 100 mm petri dishes (5×10^5 cells/plate) 24 h before transfection. Cells were transfected with pcDNA3/RPTPα(*wt*) or with pcDNA3/neo (control) using a modified calcium phosphate-mediated transfection protocol. For stable transfection cells were trypsinized 48 h after transfection, and plated in the presence of G418 (500 µg/ml). Single colonies were picked and expanded.

Colony assays

MCF-7 cells were plated in 100 mm petri dishes (5×10^5 cells/ plate) 24 h before transfection. Cells were transfected with pcDNA3/RPTPα(*wt*) or control vector pcDNA3/neo in duplicate by using lipofectin-mediated gene transfer, using the manufacterer's instructions (Life Technologies, Inc. Gaithesburg, MD). After 3 weeks of

selection in 500 µg/ml of G-418, plates were stained using Diffquick kit (Baxter, Dudingen, Switzerland).

Proliferation assay

MCF-7 parental cells and transfected MCF-7 cells were seeded in 96-well plates. Every day cells were fixed by incubating for 1 h at 4 °C in ice cold 10% TCA. Cells were then washed with PBS, and incubated for 30 min. with 0.4% Sulphorodamine B (SRB) in 1% acetic acid (100 µl/well). After three washes in 1% acetic acid, the dye was dissolved in 10 mM Tris-HCl pH 10.5 (100 µl/well) and spectrophotometrically evaluated at 492 nm [41].

Src kinase assay

Cell lysates were subjected to immune precipitation with anti-src monoclonal antibody 327 (Oncogene Science). Immune complexes were washed three times with RIPA buffer, and one time with kinase buffer (20 mM HEPES pH 7.0, 10 mM $MnCl_2$). One half of each immunoprecipitate was incubated in a volume of 50 µl kinase buffer containing 5 µCi of γ-^{32}P-ATP and 12.5 µg acid-denatured enolase at 37 °C for 5 min. The reaction was stopped by adding an equal volume of gel loading buffer and the proteins were resolved by 10% SDS-PAGE followed by autoradiography. The other half of each immunoprecipitate was run on 10% SDS-Page, and subjected to immunoblotting with monoclonal antibody 327 to measure the amount of c-Src protein.

Cell cycle analysis

Cell cycle analysis was performed on parental MCF-7 cells, a pcDNA3/neo transfected (control) clone, and three pcDNA3/RPTPα(*wt*)-transfected clones. Exponentially growing cells were trypsinized and collected in PBS. After 15 min of fixation in 50 % methanol, cells were incubated at 37 °C for 30 min. in the presence of ribonuclease A (1 mg/ml; Sigma). DNA staining was performed at 0 °C by 30 min. incubation with propidium

iodide (50 μg/ml; Sigma) in PBS containing 0.03 % bovine serum albumin. Fluorescence was measured using a FACScan flow cytometer and data were analyzed using CellFIT Software (Becton-Dickinson, Mountain View, CA).

RESULTS

High levels of RPTPα protein are found in a subset of human breast tumors

51 cases of primary breast carcinoma were analyzed for RPTPα expression by immunoblotting of total protein lysates extracted from samples obtained at surgery. This analysis revealed the presence of a single protein species of 130 kDa that cross-reacted with an anti-RPTPα antiserum raised against the entire intracellular domain of the protein (Fig.1). In transient transfection experiments in 293 cells, this antibody showed a marginal cross-reactivity with RPTPε, the PTPase which is most closely related to RPTPα in its intracellular domain (data not shown). Additional tests were therefore performed to exclude possible cross-reactivity of the antibody with RPTPε. To this purpose, samples were analyzed by immunoprecipitation using antiserum 210, directed against an epitope which is not conserved between RPTPα and RPTPε, followed by immunoblotting with antiserum 35, against the C-terminus of RPTPα. This Western blot analysis revealed that whereas only marginal levels of RPTPα could be detected in nortmal tissue or in the majority of tumors, the expression of RPTPα is substantially increased in 11 cases out of the 51 analysed (27%).

RPTPα expression correlates with low-grade tumors

We subsequently analyzed the correlation between RPTPα overexpression and pathological, clinical and biological parameters. No differences in the frequency of RPTPα expression were observed between ductal and lobular carcinoma samples (data not shown).

However, as shown in Table 1, the tumors overexpressing RPTPα were found to be of significantly lower grade (p=0.02) [37], and displayed a tendency towards ER-negativity, smaller size, and negative proliferation index [38].

High RPTPα expression in human tumors is not due to gene amplification but correlates with enhanced mRNA levels

In order to investigate if the overexpression of RPTPα was due to gene amplification, we performed Southern Blot analysis on genomic DNA extracted from the primary tumors. 10 cases that resulted positive for RPTPα overexpression (as revealed by immunoblotting), one case with no overexpression, and one normal mammary tissue sample were analyzed using two different probes corresponding to the extracellular region of RPTPα. The results, shown in Fig 2, indicate that none of the cases under study displayed RPTPα gene amplification.

In the absence of gene amplification as a plausible mechanism (fig. 2A), we subsequently performed Northern analysis on RNA from a subset of the tumors analyzed in table 1, so as to determine whether differences in RPTPα protein expression between tumors might reflect altered RPTPα mRNA levels. Out of 4 tumors displaying elevated mRNA protein levels analzyzed in this manner, we observed a higher level of RPTPα mRNA in 3, whereas no differences in RPTPα mRNA were observed in 4 negative cases (data not shown). This result indicates that enhancement of mRNA levels is likely to be the primary mechanism behind the enhanced RPTPα protein levels in primary tumors. Of note, enhanced RPTPα mRNA levels were also observed in a subset of primary human colon cancer cases [29].

RPTPα overexpression in mammary cells leads to growth inhibition.

The effect of RPTPα expression on growth parameters was investigated in the human breast cancer cell line MCF-7. In one experimental approach, an RPTPα expression vector (or the corresponding empty control vector) was transfected into MCF-7 cells, followed by a 3 weeks selection period. Individual clones were isolated and tested for RPTPα expression by anti-RPTPα immunoblotting (fig.3a). Three different RPTPα overexpressing clones were then randomly chosen for subsequent studies. Consistent with

what has been reported in other cell lines [30,31], increased RPTPα expression resulted in elevated c-src kinase activity, as measured by *in vitro* kinase assay in c-src immune precipitates vis-a-vis enolase as an exogenous substrate (fig. 3b), presumably due to the specific dephosphorylation of the negative regulatory site Y527 by RPTPα. Analysis of the *in vitro* growth properties of the clones by Sulphorodamine B proliferation assay [41] revealed that the growth rate of the four clones was significantly decreased by RPTPα expression, in comparison with that of parental or empty vector-transfected cells (fig 3c). Cell proliferation rates were also investigated by cell cycle analysis. After propidium iodide staining of cells at 50% confluence, the cell cycle distribution was deducted by flow cytometry. Fig. 3d shows a representative experiment, in which the results obtained for one control transfected clone and two RPTPα-overexpressing clones (R10 and A9) are indicated. In three independent experiments, the proportion of cells in G0/G1 phase was in the range of 35-40% for parental cells or empty transfected cells; by contrast, for RPTPα-overexpressing clones, the proportion of cells in this phase was increased to 62-78 %. The proportion of cells in S-phase was also reduced in RPTPα overexpressing cells as compared to the control cells: for parental cells and empty transfected cells, values in the range of 40-44% were found, whereas in RPTPα-overexpressing clones the proportion of cells in S-phase was decreased to 31-13%. It is well known that the appearance of a hypodiploid peak represents a specific maker of apoptosis. The absence of any sub-G1 peak after propidium staining in our cell cycle analysis suggests that the growth inhibition is not accompanied by apoptotic cell death in RPTPα-overexpressing clones. No apoptotic peaks were revealed in this analysis.

RPTPα expression reduces tumor growth and metastasis

The human MCF-7 line is only poorly tumorigenic when inoculated into immuno-deficient test animals. Therefore, to analyze the consequences of RPTPα overexpression for tumor growth *in vivo*, we expressed the RPTPα protein in murine N202-1A cells, a mouse line

derived from a mammary tumor induced by transgenic expression of the HER2/neu protooncogene in the mammary epithelium [40]. N202.1A cells were infected *in vitro* with either a retrovirus encoding for RPTPα, or (as a control) virus corresponding to the empty retroviral vector. Cells growing out after infection with either virus and the corresponding selection were pooled, so as to neutralize any effects of random clonal variability. Western Blot analysis performed on the total lysates of pooled cells confirmed the presence of RPTPα in cells infected with RPTPα encoding retrovirus compared to the control-infected cells (Fig 4a). Both cell pools were then inoculated into 6-week old Balb/c athymic mice with the aim to monitor the effect of RPTPα expression on tumor growth (following subcutaneous injection) or metastatic ability (following intravenous injection). As shown in Fig. 4b. tumor growth in animals injected subcutaneously with RPTPα-expressing cells was significantly reduced as compared to animals injected with the control cell population. In a separate experiment, to monitor development of lung metastases, control or RPTPα-expressing cells were injected intravenously, and lung metastasis were monitored by necropsy after animals showed dispnea. All the mice injected with control infected cells developed lung metastases within 45 days, whereas 4 mice injected with the RPTPα-tranfected cells developed lung metastasis within 55 days, with one remaining tumor-free (Fig 4c).

DISCUSSION

In this paper we report on the observations that RPTPα overexpression occurs in almost one third of primary human breast carcinomas, and that this phenomenon is associated with lower-grade tumors . To our knowledge, this is the first example of a study correlating expression level of a *bona fide* protein tyrosine phosphatase with neoplastic disease status. Furthermore, our experiments on breast cancer cells transfected with the RPTPα gene show that enhanced RPTPα expression lead to reduced growth rates *in vivo* and *in vitro*, an experimental effect not inconsistent with the observation on human tumors.

That enhanced RPTPα expression correlates with reduced tumor aggressiveness, and reduces both the growth rate *in vitro* and the tumorigenicity of transfected cells was somewhat unexpected. The ability of RPTPα to activate the kinase activity of c-Src [31,34], sometimes with transforming effects [30], rather suggested a role for RPTPα as as a potential oncogene itself, or as an enhancer of tumorigenicity. In a study of colon carcinoma, increased RPTPα mRNA expression was noted in 10 out of 14 advanced (Dukes' stage D) colon adenocarcinoma samples [29], although this study did not include less advanced tumors. A survey of various transgenic mouse models for mammary tumor development showed consistent overexpression of RPTPε, a PTPase highly related to RPTPα, in HER2/Neu-or v-Ha-Ras-induced tumors [25].

Several considerations may reconcile these two apparently conflicting sets of observations. First, RPTPα expression may activate different pathways, with different consequences, in fibroblast and in mammary epithelial cells. Similarly, the HER2/neu oncogene, is tumorigenic in 3T3 fibroblasts, [42] yet when overexpressed in MCF-7 leads to growth inhibition and differentiation [43]. Second, all human tumors analyzed in this study had reached the stage of clinically recognizable disease. Therefore, it remains possible that high RPTPα expression in a subset of tumors is a remnant of an earlier disease stage, where its expression may have contributed to initiation, or early progression, but is lost at later stages

in favor of more aggressive progression events. Third, whereas good evidence indicates that RPTPα expression activates the c-Src proto-oncogene, other, thus far unidentified targets for RPTPα may exist whose dephosphorylation leads to growth arrest. In fact, a recent study reported that the activation of Src seen in breast cancer cell lines may indeed be mediated by a phosphatase acting on Y527 in Src, but that this PTPase is different from RPTPα or several known PTPases [44]. This would again be consistent with the existence of biologically relevant RPTPα substrates other than c-Src. Ultimately, transgenic models will be needed to explore whether PTPases such as RPTPα or RPTPε can or may play a causative in tumor development, and thus whether high RPTPα expression may be the result of selective pressure for tumor development or survival.

The above considerations try to reconcile the transforming and c-Src activating potential of RPTPα with our observation that RPTPα expression preferentially occurs in a subset of low-grade tumours and inhibits tumour cell growth; yet, other interpretations are equally plausible. For instance, tumours that express RPTPα may belong to a biologically distinct subclass, for which RPTPα expression merely constitutes a marker. Primarily however, given the striking and unexpected negative effects of RPTPα expression on the growth of experimental tumors and on cell cycle distribution, it is tempting to propose that increased RPTPα expression is the result of a specific feedback response to an alteration in cellular homeostasis.

Under normal conditions, protein tyrosine phosphorylation is tightly regulated by the equilibrium between cellular PTKs and PTPases. For instance, dual-specificity phospatases that inactivate MAP kinases are induced as a consequence of the same stimuli that lead to MAPK activation [45-47]. A splicing isoform of RPTPε is expressed as a delayed early response gene in fibroblasts [48]. A perturbation of this balance, for instance as a result of a specific oncogene activation, may lead to feedback responses including the increased expression of particular PTPases, such as RPTPα. Increased expression of the PTPases LAR and PTP1B was observed to occur in human breast epithelial cell line as a

consequence of *neu* expression [49], and LAR transfection shown to suppress neu-oncogene-mediated transformation [21]. R-PTPases are also induced in many cases as a response to increased cell density, raising the possibility that this induction participates in contact inhibition [50]. Increased RPTPα expression in tumours could thus constitute a response to an as yet unidentified tumorigenic insult, or stimulus. We believe that the data presented in this paper warrant a further investigation of the concept that, that under certain conditions, PTPases in tumours act as non-constitutive, inducible tumor suppressors..

The growth inhibition seen as a result of RPTPα expression is related to cell cycle arrest in G1 in the absence of apoptosis. This observation suggests that a phosphoprotein directly or indirectly involved in cell cycle control could be a specific substrate for RPTPα. pp60v-src has been reported to be capable of inducing cyclin D1 in MCF7 cells [51]. It is unclear to what extent c-Src activation by RPTPα has similar consequences as v-Src expression, and thus the arrest-inducing effect of RPTPα may or may not be mediated through Src family kinases. A further molecular analysis of the cell cycle blocking effect of RPTPα will require novel insights ito the signaling pathways downstream of this widely expressed PTPase. The membrane localization of RPTPα can be does not exclude the possibility that this PTPase could act on substrates localized in other compartments. Protein tyrosine phosphatase epsilon (RPTPε), a protein of the same family of RPTPα, exists in both cytoplasmic and trans-membrane forms [48]. Furthermore, the cleavage of the extracellular domain of membrane phosphatases was shown to induce the cellular redistribution of the catalytic domain [52].

One tempting clue to the mechanism behind the ablity of RPTPα to block cell cycle progression *in vitro* and tumor growth *in vivo* is provided by the ability of RPTPα to block insulin responsiveness; this effect has now been observed in a large number of cellular environments and insulin response parameters: anti-adhesive effects of insulin in BHK cells [53], prolactin promoter activation in GH4 pituitary cells [54], and GLUT4 translocation in primary adipocytes [55]. However, the identity of the target of RPTPα in

the insulin signaling pathways is as yet unclear. Insulin-like growth factors are clearly implicated in progression through the cell cycle, proliferation, and inhibition of apoptosis of breast cancer cells *in vitro* [7,56,57]. Moreover, fibroblasts lacking the IGF-1 receptor are resistant to transformation by dominant acting oncogenes [58,59]. The ensuing hypothesis, that the potential of RPTPα to counteract the effects of insulin-like growth factors relates to the inverse correlation between RPTPα expression and mammary tumor growth or malignancy, is readily testable.

Parameter	RPTPα not overexpressed	RPTPα overexpressed
Number of cases:	36	15
Age > 55years:	44% (16/36)	47% (7/15)
Tumor grade III:	53% (18/34)	20% (3/15) (p=0.02)
grade II:	47% (16/34)	73% (1/15)
grade I:	0% (0/30)	7% (1/15)
Necrosis:	50% (18/36)	47% (7/15)
Proliferation index positive:	32% (11/34)	15% (2/13) (p=0.15)
Size (<2 cm):	14% (5/35)	33% (5/15) (p=0.09)
N+	81% (29/36)	67%(10/15) (p=0.15)
1-4:	41% (12/29)	50% (5/10)
5-8:	28% (8/29)	40% (4/10)
>8:	31% (9/29)	10% (1/10) (p=0.15)
ER negative:	37% (13/35)	20% (3/15) (p=0.04)
PgR negative:	54% (19/35)	46% (7/15)
HER2/neu positive:	43% (9/21)	17% (2/12) (p=0.10)

Table 1: Pathological parameters of RPTPα-positive versus -negative human breast tumors. Tumor grade is according to [36]. Positive proliferation index is defined as previously reported, with 2.3% of labeled cells being the cut-off value [38]. N+; number of metastasis-containing lymph nodes. ER: Estrogen receptor. PgR: progesterone receptor.

FIGURE LEGENDS

Fig. 1. Expression of RPTPα protein in human breast tumors. Total protein lysates were subjected to anti-RPTPα immunoblotting, using an antiserum raised against the intracellular domain of RPTPα.

Fig. 2. Southern blotting analysis of tumor DNA from a set of human breast tumors (T) or one instance of neigbouring normal tissue (N). All Tumors overexpressed RPTPα, except 16289. Extracted tumor DNA was digested with EcoRI, separated by agarose gel electrophoresis, and analyzed with a radioactive probe corresponding to sequences in the extracellular domain of RPTPα.

Fig. 3. Expression of RPTPα inhibits *in vitro* growth rate due to extension of G1 phase of the cycle.

A. Immunoblotting analysis of expression of RPTPα protein in stably transfected MCF-7 cell clones.

B. Activation of c-Src kinase activity as a result of RPTPα expression. Top panel: c-Src protein was immune precipitated and subjected to an *in vitro* kinase reaction in the presence of γ-^{32}P-radiolabeled ATP, using enolase as exogenous substrate. Bottom panel: an aliquot of the Src immune precipitate was subjected to anti-Src immunoblotting.

C. Effect of RPTPα expression on growth rate. Equal numbers of parental MCF-7 cells (circles), a vector (control) transfected MCF-7 cell clone (squares), and three RPTPα-expressing clones (triangles) were seeded (in DMEM containing 10 % fetal calf serum) at equal initial densities, and growth measured using the Sulphorodamine B dye uptake method [41].

D. Cell cycle distribution of control and RPTPα expressing cells. Exponentially growing cells were trypsinized, fixed, and stained with propidium iodide, and fluorescence measured by flow cytometry. A=control cells; B,C=RPTPα-expressing cells.

Fig. 4. Expression of RPTPα affects tumor growth *in vivo*. Murine N202.1A cells (derived from a Neu-induced mammary tumor)[40] were infected with empty control (squares), or with an RPTPα-expressing retrovirus (triangles). After selection, stably expressing pools of the respective cells were inoculated using the appropriate routes into nude mice.

A: Immunoblot analysis of RPTPα expression; B: Tumor volume following subcutaneous injection; C: Development of lung metastasis following tail vein injection.

Squares=control (vector transfected cells); triangles=RPTPα-expressing cells.

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Fig. 1

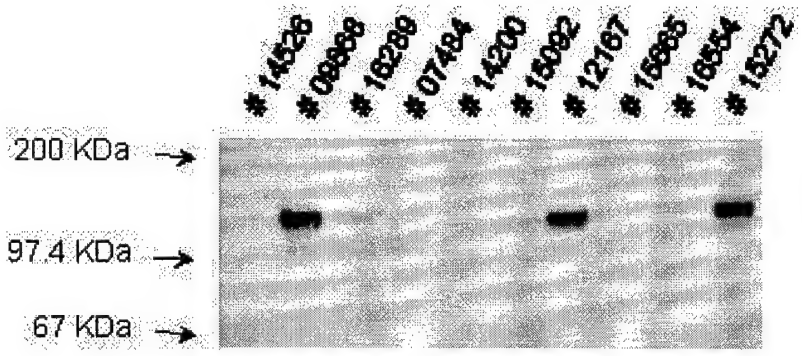
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Fig. 2

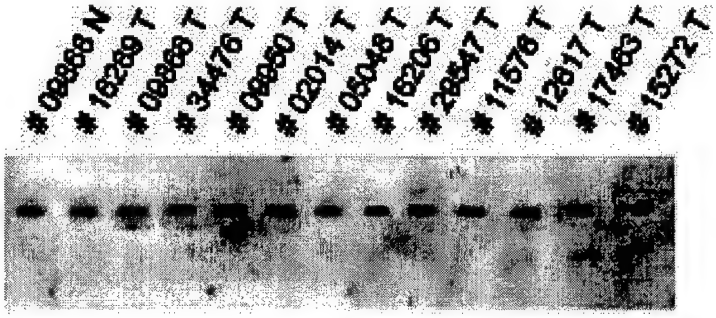
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Figure 3A

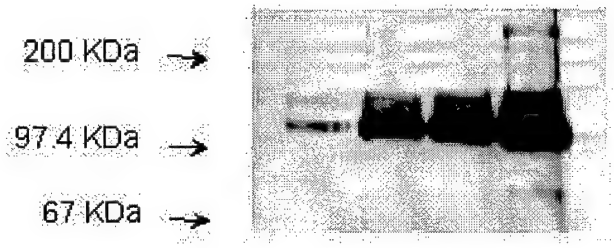


Figure 3B

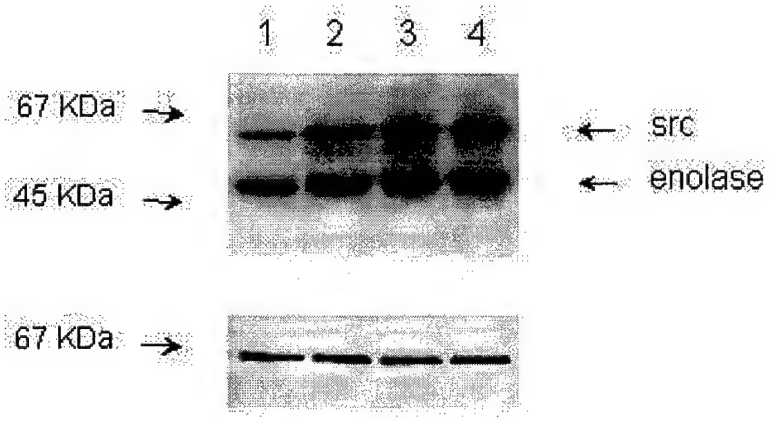
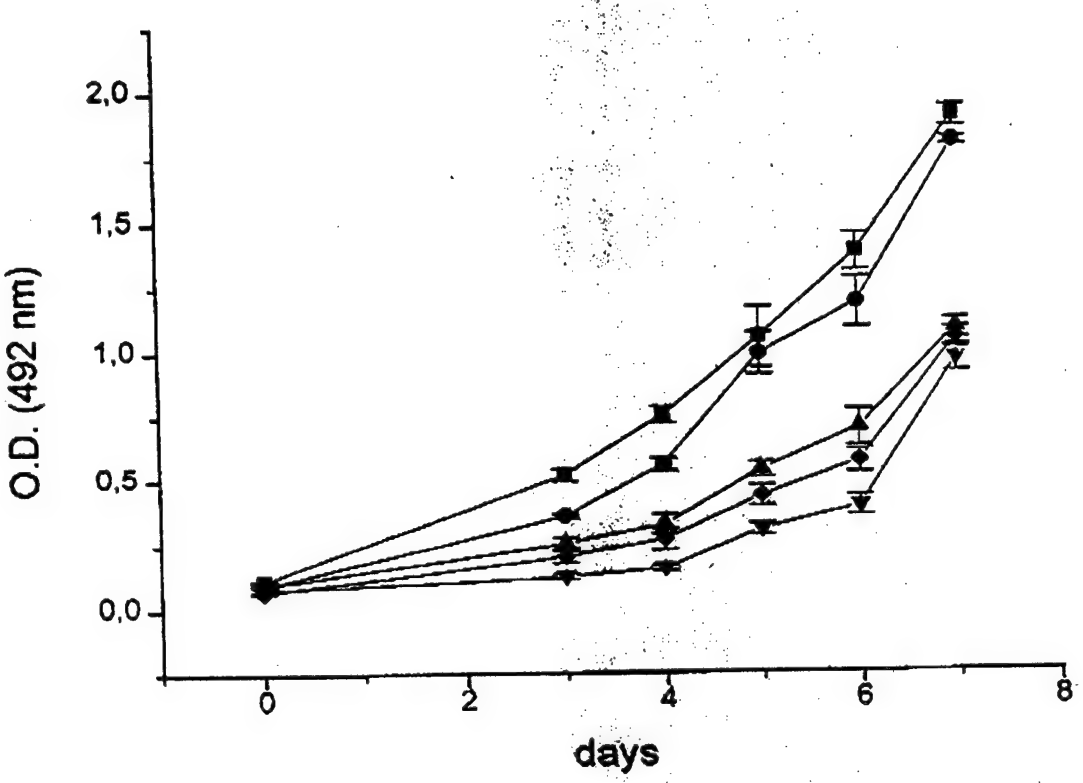


Figure 3C



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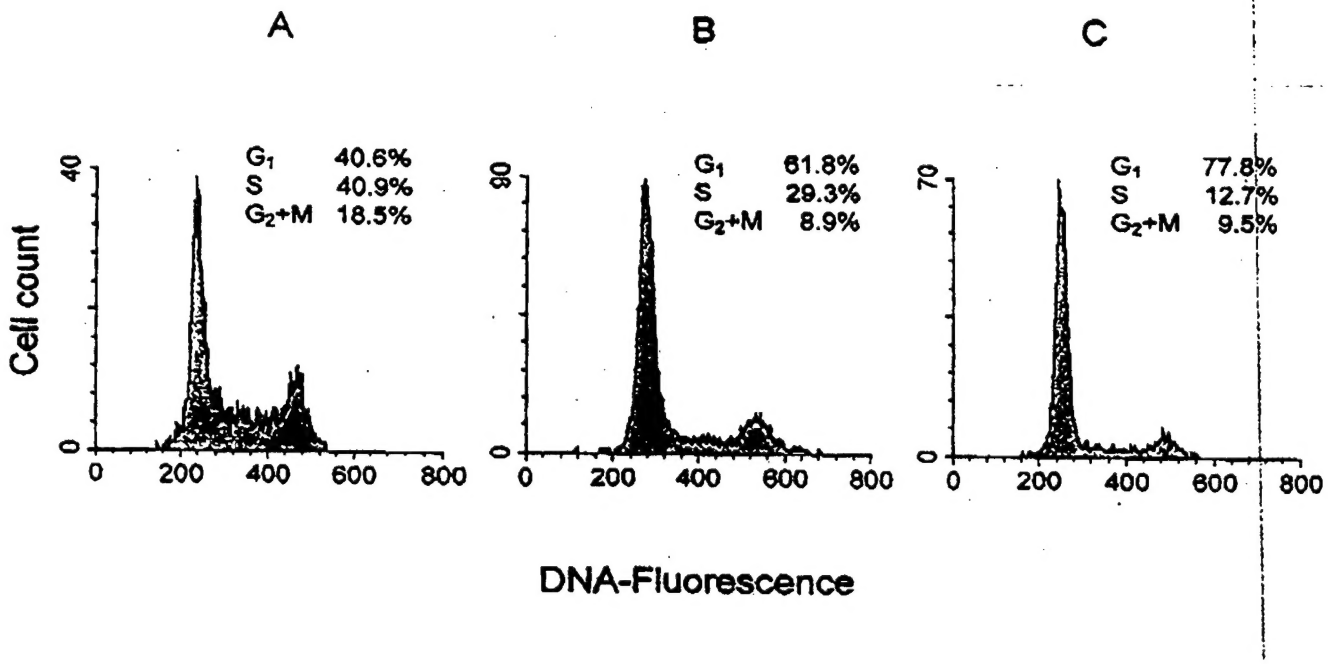
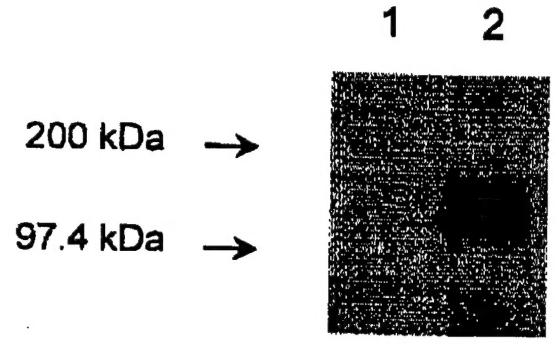

A
B
C
G1 40.6%
S 40.9%
G2+M 18.5%
G1 61.8%
S 29.3%
G2+M 8.9%
G1 77.8%
S 12.7%
G2+M 9.5%
Cell count
DNA-Fluorescence
0
40
90
70
200
400
600
800

Figure 4A



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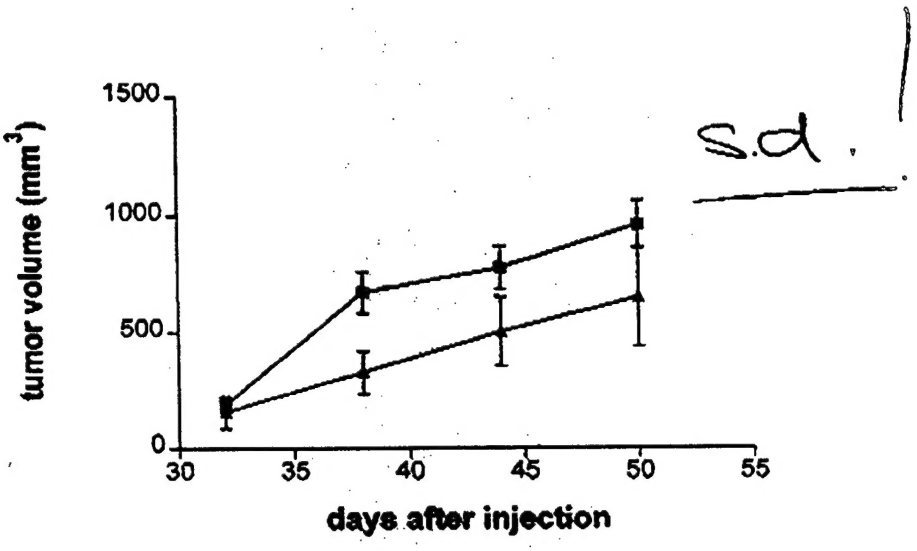
1500
1000
500
0
tumor volume (mm3)
30
35
40
45
50
55
days after injection
S.d.

Figure 4C